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Transcriptional regulation of the ORF61 and ORF60 genes of Kaposi's sarcoma-associated herpesvirus

Shie-Shan Wang [a,b,1], Lee-Wen Chen [e,1], Li-Yu Chen [a], Hsin-Hwa Tsai [a], Yan-Chung Shih [a], Cheng-Ta Yang [c], Pey-Jium Chang [a,d,*]

[a] Graduate Institute of Clinical Medical Sciences, Chang-Gung University, Taoyuan, Taiwan
[b] Department of Pediatric Surgery, Chang-Gung Memorial Hospital at Chiayi, Taiwan
[c] Department of Internal Medicine, Chang-Gung Memorial Hospital at Chiayi, Taiwan
[d] Department of Medical Research, Chang-Gung Memorial Hospital at Chiayi, Taiwan
[e] Department of Respiratory Care, Chang-Gung Institute of Technology, Chiayi, Taiwan

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ABSTRACT

The ORF61 and ORF60 genes of Kaposi's sarcoma-associated herpesvirus (KSHV) encode the ribonucleotide reductase large and small subunits, respectively. Here we show that ORF50 protein, a latent-lytic switch transactivator, activates the transcription of these two early-lytic genes through different mechanisms. Activation of the ORF61 promoter by ORF50 protein is dependent on an intact RBP-Jκ-binding site within the identified responsive element and the expression of RBP-Jκ protein in cells. The critical element in the ORF60 promoter in response to ORF50 was mapped to a 40-bp region. Binding of YY1, Sp1/Sp3 or unknown proteins to this element may contribute to repression or activation of the ORF60 promoter. Although ORF50 protein does not directly bind to the ORF61 and ORF60 promoters *in vitro*, we show the association of ORF50 protein with these two promoters *in vivo*. Our results provide further insights into the regulatory network of the viral lytic genes in KSHV reactivation.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is a gammaherpesvirus associated with at least three malignancies: Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease (Boshoff et al., 1995; Cesarman et al., 1995; Chang et al., 1994; Soulier et al., 1995). An important step for viral propagation and pathogenesis is the switch from latency to lytic replication (Martin et al., 1999; Miller et al., 1997). Upon reactivation from latency, the viral lytic genes are expressed in a temporally ordered fashion, ultimately leading to the production of infectious virions (Gradoville et al., 2000). The lytic genes can be classified into three categories: immediate-early, early and late genes (Miller et al., 1997; Sun et al., 1999). The immediate-early genes usually encode regulatory proteins that modulate expression of viral and host genes, which facilitate later stages of lytic replication (Zhu et al., 1999). Following expression of the immediate-early genes, the early genes that encode essential components for viral lytic DNA synthesis are induced. In addition to the replication proteins, the KSHV early gene products also include transcriptional modulators, signal transducers, cytokines and immunomodulatory proteins (Dourmishev et al., 2003). After lytic DNA synthesis, viral capsid proteins or structural glycoproteins are expressed at late stages. Expression of these lytic genes in different categories must be tightly controlled during the entire lytic cycle.

The ORF50 protein encoded by open reading frame 50 (ORF50) of the viral genome is an immediate-early protein, which is a key regulator for the switch of the virus from latency to lytic replication (Lukac et al., 1999, 1998; Sun et al., 1998). The ORF50 protein is a potent transcriptional activator with an N-terminal basic DNA-binding domain and a C-terminal acidic activation domain (Chang and Miller, 2004; Chang et al., 2002; Wang et al., 2001). Ectopic expression of ORF50 protein in latently KSHV-infected cells triggers the viral lytic cascade to completion (Lukac et al., 1998; Sun et al., 1998). All of the viral early genes with the similar expression kinetics must be under the control of ORF50 protein during lytic cycle. However, not all tested promoters of the early lytic genes are activated through the direct binding of ORF50 to the promoters (Chang et al., 2005; Liang et al., 2002). Thus, ORF50 protein may utilize different strategies to cooperate with other viral or cellular proteins to regulate the diverse early gene expression.

A subset of the early gene promoters activated by ORF50 have been identified, including polyadenylated nuclear (PAN) RNA, K12, ORF57,

* Corresponding author. 6 West, Chia-Pu Road, Puzi City, Chiayi, Taiwan. Fax: +886 5 3623002.
E-mail address: peyjiumc@mail.cgu.edu.tw (P.-J. Chang).
[1] These authors contribute equally to this work.

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K8, K9 (vIRF), ORF21, K3, K5, K6 (vMIP-1), ORF6, K14 (vOX-2), ORF74 (vGPCR), K2 (vIL-6), ORF59 and ORF50 itself (Chang et al., 2002, 2005; Chen et al., 2000; Deng et al., 2002, 2000; Haque et al., 2000; Jeong et al., 2001; Liu et al., 2008; Lukac et al., 2001, 1999; Song et al., 2001; Wang and Yuan, 2007; Zhang et al., 1998). Among the known targets, PAN and K12 are activated through a direct binding of ORF50 to their promoters (Chang et al., 2002). A highly conservative core element, 5′-AAATGGGTGGCTAACC, for ORF50 binding is required for the activation. Conversely, ORF50 may activate transcription through indirect access to DNA via protein-protein interaction (Chang et al., 2005; Liang et al., 2002). The best-characterized protein involving in the ORF50-mediated transactivation is RBP-Jκ (CSL/CBF1), a primary effector of the Notch pathway (Lai, 2002). The ORF50 protein interacts with RBP-Jκ *in vitro* and *in vivo* (Liang et al., 2002). Binding of the RBP-Jκ protein to the target promoters is critical for recruiting ORF50 protein to activate the transcription. Several promoters of viral genes, including the ORF57, ORF6, vMIP-1, K14/ORF74, ORF50, K3, K5, K8 and ORF59, have been grouped into this subclass (Chang et al., 2005; Liang et al., 2002; Liang and Ganem, 2003, 2004; Liu et al., 2008; Wang and Yuan, 2007). In these promoters, a conserved or related RBP-Jκ binding site, 5′-GTGGGAA, is found within the identified ORF50 response elements (ORF50 REs).

Despite extensive studies in the regulation of the early lytic genes, regulation of numerous viral lytic genes still remains elusive. As other herpesviruses, KSHV encodes the homolog of ribonucleotide reductase (RNR) that catalyzes the conversion of ribonucleoside diphosphates to the corresponding deoxyribonucleotides to provide a balanced supply of precursors for DNA synthesis (Jordan and Reichard, 1998). The KSHV RNR is composed of two subunits: the large subunit encoded by open reading frame 61 (ORF61) and the small subunit by open reading frame 60 (ORF60) (Russo et al., 1996). Since the viral RNR potentially involves the control of the cellular concentration of deoxyribonucleotides, expression of the ORF61 and ORF60 genes may be important for lytic DNA replication. In this report, we study the transcriptional regulation of these two lytic genes in response to ORF50 protein in different cell lines. The detailed regulatory networks mediated by ORF50 protein in activating the ORF61 and ORF60 promoters are proposed.

Results

Expression kinetics and transcriptional mapping of the ORF61 and ORF60 genes

To analyze the expression kinetics of the ORF61 and ORF60 genes during lytic cycle, total RNAs prepared from HH-B2 cells treated with sodium butyrate were subjected to Northern analysis. Phosphonoacetic acid (PAA), an inhibitor of lytic replication, was also added in butyrate-treated cells to differentiate the early from late transcripts. Northern analysis revealed that two transcripts that were 6.0 and 3.5 kb long were detected using an ORF60-specific probe and were expressed at maximal levels at 12 to 24 h after lytic induction (Fig. 1B). Furthermore, PAA did not seem to affect the transcription. The 6.0-kb transcript may contain ORF61, ORF60, ORF59 and ORF58; the 3.5-kb transcript may contain ORF60, ORF59 and ORF58 (Majerciak et al., 2006). To determine the location of the ORF61 and ORF60 promoters, we mapped the 5′ transcriptional start site of each gene by RACE (rapid amplification of the cDNA ends). The start site of the ORF61 transcript was mapped at nucleotide (nt) 100329 of the KSHV genome (Russo et al., 1996) (Figs. 1A and C). Whereas two transcriptional start sites, nt 97843 (major) and nt 97845 (minor), were mapped to the ORF60 transcript (Figs. 1A and C). Upstream of the identified transcriptional start sites, a consensus TATA box (5′-TATATAAA) and a TATA-like box (5′-TAAAAA) were found in the ORF61 promoter (ORF61p) and the ORF60 promoter (ORF60p), respectively (Fig. 1D). The corresponding 3′ polyadenylation cleavage sites of the ORF61 and ORF60 transcripts were predicted based on the length of the transcripts in our Northern analysis and the 3′ transcriptional mapping of the ORF58 and ORF59 genes reported previously by Majerciak et al. (2006). There were two adjacent 5′-AAUAAA signals at the 3′ end of the transcripts (Fig. 1A).

Distinct mechanisms operated by ORF50 protein in activating the ORF61 and ORF60 promoters

To determine whether ORF50 protein directly regulated the ORF61 and ORF60 promoters, regions of about 1 kb in the ORF61 and ORF60 promoters were cloned into pGL3-Basic, a luciferase reporter plasmid. Two ORF50-responsive promoters, PANp and ORF57p, were also included in the experiment (Fig. 2). The luciferase activity of these reporters in HKB5/B5, 293T and HH-B2 cells was monitored with a luminometer. Both HKB5/B5 and 293T are KSHV-negative cell lines, whereas HH-B2 is a latently KSHV-infected cell line. As illustrated in Fig. 2, the ORF50 protein strongly activated the PANp, ORF57p, ORF61p and ORF60p, 81- to 1156-fold in HH-B2 cells. In HKB5/B5 and 293T cells, the PANp, ORF57p and ORF61p were still substantially activated by ORF50 protein. However, ORF50 protein activated the ORF60p only at background levels in HKB5/B5 and 293 T cells (Fig. 2). These results indicated that ORF50 protein alone sufficiently activates the ORF61p in different cell lines. In contrast, activation of the ORF60p by ORF50 protein may require other viral or cellular proteins, which are not adequately expressed in HKB5/B5 and 293T cells.

Defining the ORF50 response element in the ORF61 promoter

Since the ORF50 protein activated the ORF61p, we determined the ORF50 response element (ORF50 RE) of the ORF61 promoter. Deletions in the ORF61p were constructed to examine their ORF50 responsiveness in HKB5/B5, 293T and HH-B2 cells (Fig. 3A). Although the ORF50 responsiveness of the ORF61 promoter in different cell lines varied, the ORF50 responsive region in the promoter was consistently mapped to the region between −344 and −123 (Fig. 3A). To define the minimal ORF50 RE in the ORF61p, the region encompassing −344 to −123 or its deleted elements (Fig. 3B) was fused to pE4luc, a luciferase reporter plasmid containing a minimal adenovirus E4 promoter. When the reporter containing the region from −344 to −123 was cotransfected with an ORF50-expressing plasmid in HKB5/B5 and 293T cells, a 16- and 46-fold enhancement of luciferase activity was detected in HKB5/B5 and 293T cells (Fig. 3B). Based on the results from Fig. 3B, the minimal ORF50 RE of the ORF61p was mapped to a 40-bp element from −194 to −155 (Fig. 3B; RE-6). Further deleting the 3′ region of the RE-6 element to −175 completely abolished the response to ORF50 protein in both cell lines, whereas 5′ deletion of the RE-6 element to −187 had a decreased ORF50 responsiveness by 22% in HKB5/B5 cells and 19% in 293T cells (Fig. 3B).

Binding of RBP-Jκ to the ORF50 RE in the ORF61 promoter

Sequence analysis of ORF61p(−194/−155) revealed that this region contained a putative RBP-Jκ recognition sequence, 5′-GTGTGAA, between −165 and −159 (Fig. 4A). To determine whether the putative RBP-Jκ site in ORF61p(−194/−155) was important to the transactivation by ORF50, point mutations were introduced into the element (Fig. 4A). Mutation at the putative RBP-Jκ site reduced the ORF50 responsiveness to background levels in HKB5/B5 and 293T cells (Figs. 4B and C; F-mt). Mutations flanking the RBP-Jκ site of the ORF61p (−194/−155) element also resulted in reduction of their ORF50 responsiveness by 26% to 60% in HKB5/B5 cells and 46% to 64% in 293T cells (Fig. 4B and C), suggesting that the region flanking the RBP-Jκ recognition site is also critical for the full ORF50 responsiveness.

To determine the binding of ORF50 or RBP-Jκ protein to the ORF61p(−194/−155) element, extracts from HKB5/B5 cells transfected with pCMV, pCMV-FLAG-ORF50, pCMV-FLAG-ORF50(1–390)

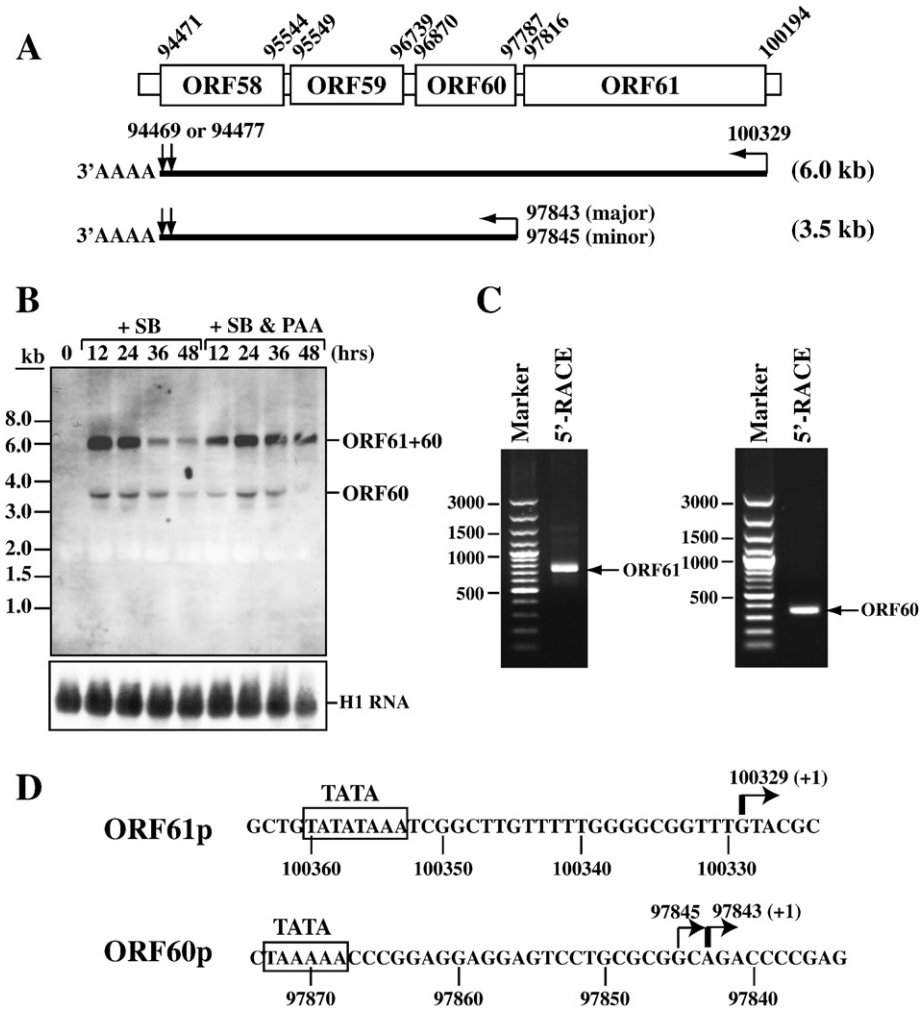

Fig. 1. Expression and determination of the transcription start sites of the ORF61 and ORF60 genes. (A) Schematic diagram of the ORF61 and ORF60 transcripts. Numbers in the diagram represent the nucleotide positions of the KSHV genome (Russo et al., 1996). Arrows represent transcriptional start and cleavage sites. (B) Northern blot analysis of the ORF61 and ORF60 transcripts. Total RNAs were prepared from HH-B2 cells that were treated with 3 mM sodium butyrate (SB) for 12, 24, 36 and 48 h in the presence or absence of 200 μg phosphonoacetic acid (PAA) per ml. The DNA fragment from nt 97527 to 97843 was used as a probe for Northern analysis. Hybridization with H1 RNA of RNase P served as a loading control. (C) Mapping of the transcriptional start sites of the ORF61 and ORF60 transcripts by RACE. Total RNAs from HH-B2 cells treated with sodium butyrate for 30 h were subjected to RACE analysis as described in Materials and methods. The 5′ regions of the ORF61 and ORF60 transcripts amplified in RACE experiments were shown. (D) Sequence of the basal promoter of the ORF61 and ORF60 genes. Arrows and the open boxes represent the transcriptional start sites and the TATA boxes, respectively. The transcriptional start site of the ORF61 gene was mapped to nt 100329, whereas the transcriptional start site of the ORF60 gene was mapped to nt 97843 (major) or nt 97845 (minor).

or pCMV-FLAG-RBP-Jκ were used in an EMSA study. The FLAG-ORF50 (1–390) protein contains only the DNA-binding domain from aa 1 to 390 of ORF50 protein. The study found that FLAG-RBP-Jκ protein, but not the full-length ORF50 and ORF50(1–390), bound to the ORF61p (−194/−155) probe (Fig. 4D). The DNA/RBP-Jκ complex formed in EMSA was also confirmed by supershifting the complex with an anti-FLAG antibody (Fig. 4D). As shown in Fig. 4E, formation of the DNA/RBP-Jκ complex was blocked with excessive competitors RE-6, M-mt and R-mt, but not the F-mt cold competitor. The data suggested that the RBP-Jκ protein binds to the 5′-GTGTGAA sequence. Furthermore, when the RBP-Jκ recognition site, 5′-GTGTGAA, in the ORF61p(−194/−155) element was converted to a conserved RBP-Jκ site, GTGGGAA [Fig. 4A; RBP(+)], the single point mutation present in the ORF61p (−194/−155) element exhibited 1.6- and 1.5-fold greater ORF50 responsiveness than the wild-type RE-6 element in HKB5/B5 and 293T cells [Figs. 4B and C; RBP(+)].

Involvement of the RBP-Jκ protein in activating the ORF61 promoter

Next, we examined the role of RBP-Jκ protein in activation of the ORF61p by ORF50 protein. The embryonic fibroblast cell line OT11 (kindly provided by Dr. T. Honjo, Kyoto University, Kyoto, Japan) derived from the RBP-Jκ knockout mouse was used to analyze the ORF50 transactivation. The parental wild-type mouse cell line OT13 was also used as a control for analyzing the ORF50 transactivation. The ORF61p and PANp revealed a 61- and 121-fold enhancement of luciferase activity in OT13 cells (Fig. 4F). However, little response of the ORF61p to ORF50 protein was detected in OT11 cells (Fig. 4G). Notably, the PANp still conferred strong ORF50 responsiveness (48-fold) in OT11 cells. When the plasmid expressing RBP-Jκ was introduced into OT11 cells, activation of the ORF61p by ORF50 was restored (Fig. 4G), indicating that ORF50 activates the ORF61 promoter though the RBP-Jκ protein.

Defining the ORF50 regulatory element in the ORF60 promoter

Although the ORF60p was not a direct target of ORF50 protein, activation of the ORF60p remained under the control of ORF50 protein in the KSHV-infected cells (Fig. 2). To investigate the regulatory network that influences the transcription from this promoter, we determined the location of the ORF50 regulatory element in the ORF60p in HH-B2 cells. A series of 5′ deletions of the ORF60p were

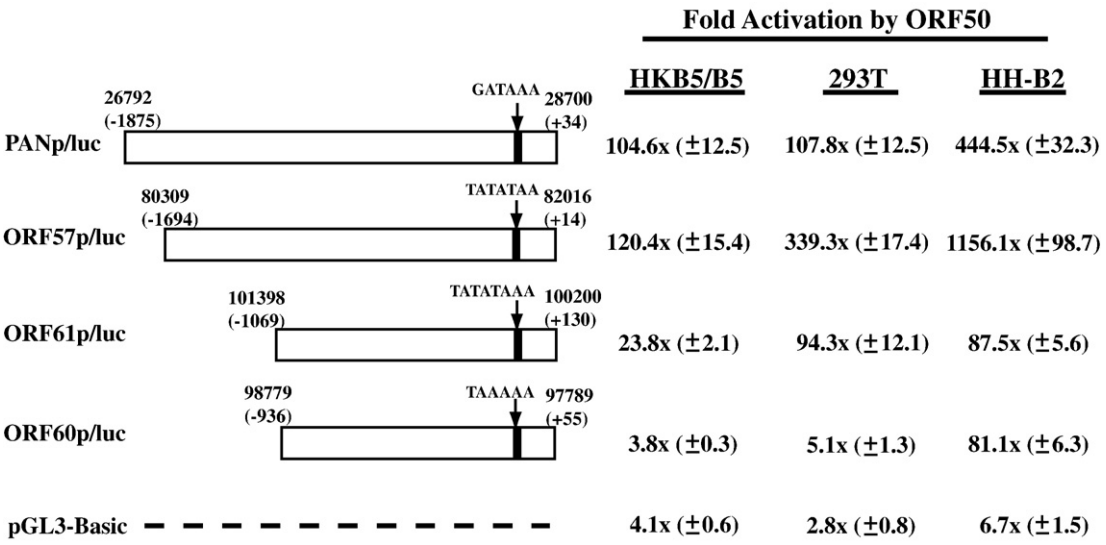

Fig. 2. Transcriptional activation of viral promoters by the ORF50 protein in different cell lines. The promoter regions from the PAN and ORF57, ORF61, ORF60 genes were cloned into pGL3-Basic. The reporter plasmids were individually cotransfected with pCMV or pCMV-FLAG-ORF50 into HKB5/B5, 293T and HH-B2 cells. At 48-h post-transfection in HKB5/B5 and 293 cells or at 24-h post-transfection in HH-B2 cells, the luciferase activity of the reporters was measured. The fold activation was calculated as the luciferase activity in the presence of the ORF50-expressing plasmid divided by luciferase activity in the presence of control vector pCMV. The means and standard deviations from at least three independent transfections are shown.

generated to examine their response to ORF50 protein (Fig. 5A). Transient reporter assay revealed that 5′ deletion to −70 relative to the transcription start site retained nearly full responsiveness to the ORF50 protein in HH-B2 cells. However, the response was lost when the deletion was extended to −31 (Fig. 5A). To further confirm the importance of the regulatory element responding to the ORF50 protein in the ORF60p, the region from −71 to −−32 of the ORF60p was subcloned into pE4luc. This 40-bp sequence in pE4luc conferred 37-fold activation in response to ORF50 in HH-B2 cells (Fig. 5B). Point mutations were also generated in this 40-bp regulatory element. Mutation in the middle region of the regulatory element led to background activation by ORF50 protein, whereas mutations at the flanking region also resulted in reduction of the ORF50 transactivation (Fig. 5B).

Binding of YY1, Sp1/Sp3 and unknown proteins to the ORF60 promoter

To determine the binding capacity of cellular and viral proteins to the ORF60p(−71/−32) element, extracts from HH-B2 cells that were treated or untreated with sodium butyrate were used in EMSA experiments. Two major DNA/protein complexes, designated A1 and A3, were detected in EMSA using the untreated cell extract, whereas an extra DNA/protein complex A2 was present if the butyrate-treated extract was used (Fig. 6A). Notably, formation of the complex A1 and A3 in EMSA using the butyrate-treated cell extract was less abundant than that using the untreated cell extract (Fig. 6A). By using excessive cold competitors in EMSA, we found that the protein components of the complex A1 and A3 specifically bound to the middle region in the regulatory element (Fig. 6A). By contrast, the protein component of the complex A2 displayed nonspecific-binding feature to the regulatory element. Sequence analysis revealed that a consensus YY1 binding site, 5′-CCATNTT (Hyde-DeRuyscher et al., 1995), and an Sp1 (or Sp3) binding site, 5′-GGGGCGTGG (Li et al., 2004), were present in the middle region of the regulatory element (Fig. 5B). To determine whether ORF50 or these cellular proteins bound to the ORF60p(−71/−32) element, antibodies specific to ORF50, YY1, Sp1 and Sp3 were used to supershift the complexes formed in EMSA. Addition of the anti-ORF50 antibody did not affect the formation of the DNA/protein complexes in EMSA (Fig. 6B). However, either monoclonal or polyclonal YY1 antibody was sufficient to remove the complex A3, suggesting that YY1 protein was an important component of the complex A3. After removal of the YY1 complex, we found a faint DNA/protein complex (A3′), which co-migrated with the complex A3, detected only in EMSA using the extract from butyrate-treated HH-B2 cells (Fig. 6B). When the Sp1 and Sp3 antibodies were used in the reaction, the complex A1 formed in EMSA was partly reduced (Fig. 6B). To further confirm whether ORF50, YY1 or Sp proteins bound to the regulatory element of the ORF60 promoter, cell extracts containing the overexpressed FLAG-ORF50, FLAG-ORF50(1–390), FLAG-YY1 or FLAG-Sp1(300–785) were used in EMSA. The FLAG-Sp1(300–785) contains the DNA-binding domain of Sp1 (Li et al., 2004). As shown in Fig. 6C, YY1 and Sp1(300–785), but not full-length ORF50 and ORF50(1–390), bound to the ORF60p(−71/−32) element. These results imply that different cellular (or viral) proteins bound to the ORF60p under latent or lytic condition may contribute to repress or activate the gene expression.

Down-regulation of YY1, Sp1 and Sp3 during KSHV lytic cycle

The reduced binding of YY1 and Sp1/Sp3 proteins to the ORF60p (−71/−32) element in lytic condition shown in Fig. 6 prompted us to determine whether the expression of YY1, Sp1 and Sp3 proteins was affected during KSHV lytic replication. Protein extracts from HH-B2 cells treated with sodium butyrate for 24 and 48 h were prepared and subjected to Western blot analysis. As expected, two viral lytic proteins, ORF50 and K8, were induced at 24 h after sodium butyrate treatment (Fig. 7A). However, the expression of YY1, Sp1 and Sp3 was reduced in a time-dependent manner (Fig. 7A). Furthermore, the KSHV lytic cycle was also induced in a cell line HH-B2(Tet-On-F-ORF50) in which expression of exogenous ORF50 gene is regulated under the control of the doxycycline-induced promoter. Similar to that in the parental HH-B2 cells after butyrate treatment, expression of YY1, Sp1 and Sp3 proteins in HH-B2(Tet-On-F-ORF50) was reduced in a time-dependent manner after viral lytic induction with doxycycline (Fig. 7B).

Repressive effect of YY1 on the ORF60p activation

Due to the presence of the YY1 binding site in the ORF60p(−71/−32), we examined the effects of the overexpressed YY1 on the

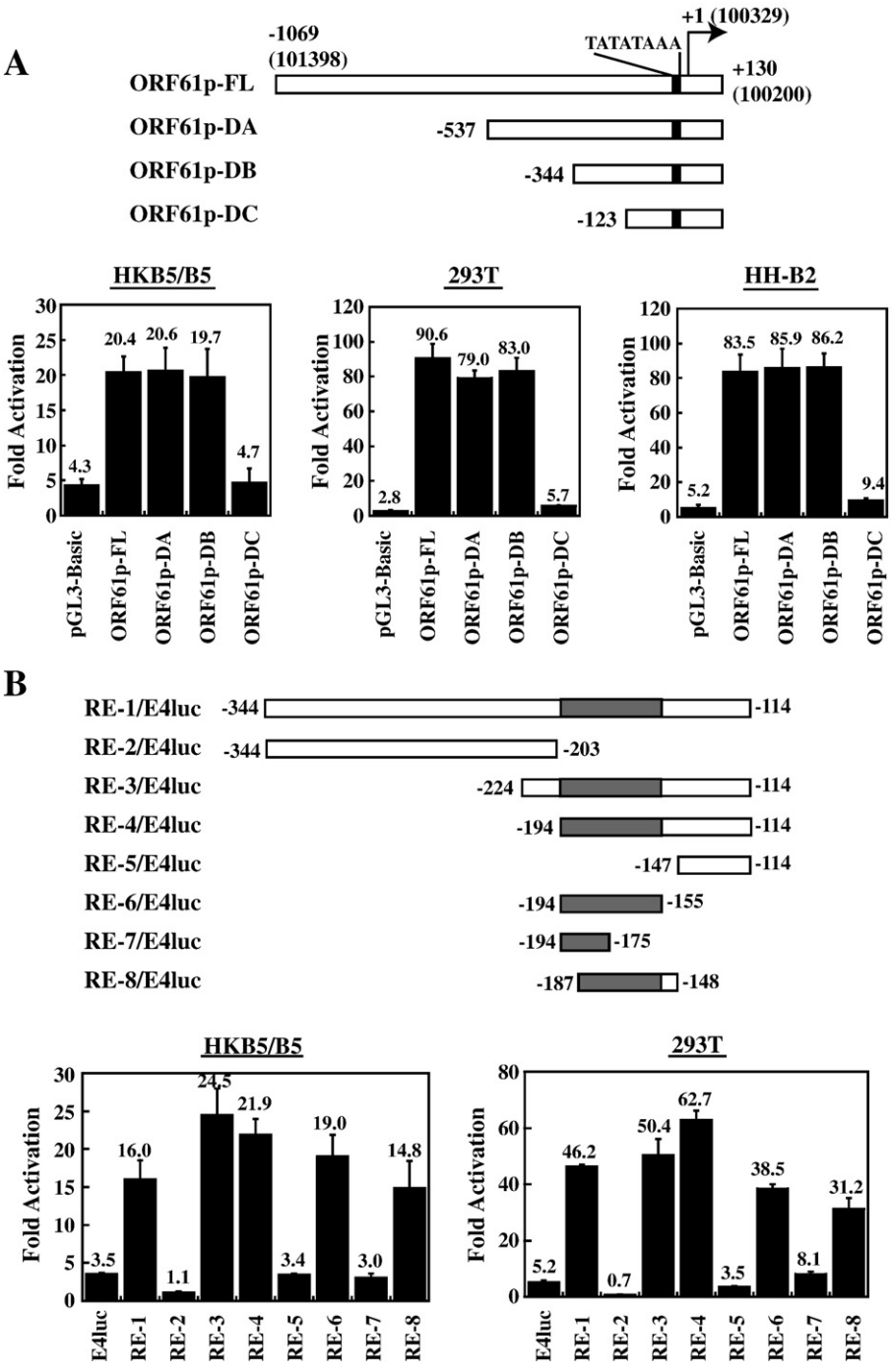

Fig. 3. Defining the ORF50 response element (ORF50 RE) of the ORF61 promoter. (A) Responsiveness of 5′ deletion mutants of the ORF61 promoter to the ORF50 protein in HKB5/B5, 293T and HH-B2 cells. Activation of the 5′ deleted ORF61 promoter by ORF50 protein in cells was determined by luciferase assay and activation fold was calculated as described above. (B) Fine mapping of the ORF50 RE within the ORF61 promoter. The ORF61p elements shown in the diagram were cloned into pE4luc, a luciferase reporter plasmid containing the adenovirus minimal E4 promoter. The reporter activation of each construct in response to ORF50 protein was determined in HKB5/B5 and 293T cells.

activation of the ORF60p(−71/−32)/E4luc by ORF50. Two other reporter plasmids that contain the E2F-binding site and the ORF61p (−194/−155) element in pE4luc were also included in the experiment. Increasing amounts of the YY1-expressing plasmid were cotransfected with the reporters and the ORF50-expressing plasmid into HH-B2 cells. At 24-h post-transfection in HH-B2 cells, the luciferase activity of the reporters was measured. The pE2F/E4luc exhibited a background level activation by ORF50 and was weakly repressed by YY1 (Fig. 8A). However, the increasing amounts of YY1 significantly repressed the activation of the ORF60p(−71/−32)/E4luc by ORF50 in a dose-dependent manner (Figs. 8B and D). Little repressive effect was detected for the reporter ORF61p(−194/−155)/E4luc in the presence of the overexpressed YY1 (Figs. 8C and D).

Binding of ORF50 protein to the ORF61 and ORF60 promoters in vivo

Association of ORF50 and RBP-Jκ with the viral lytic promoters was determined in HH-B2 and in HH-B2(Tet-On-F-ORF50) cells by chromatin immunoprecipitation (ChIP). Chromatin extracts of HH-B2 treated with sodium butyrate for 24 h or HH-B2(Tet-On-F-ORF50) treated with doxycycline for 24 h were immunoprecipitated with antibodies to ORF50, actin and RBP-Jκ. The association between

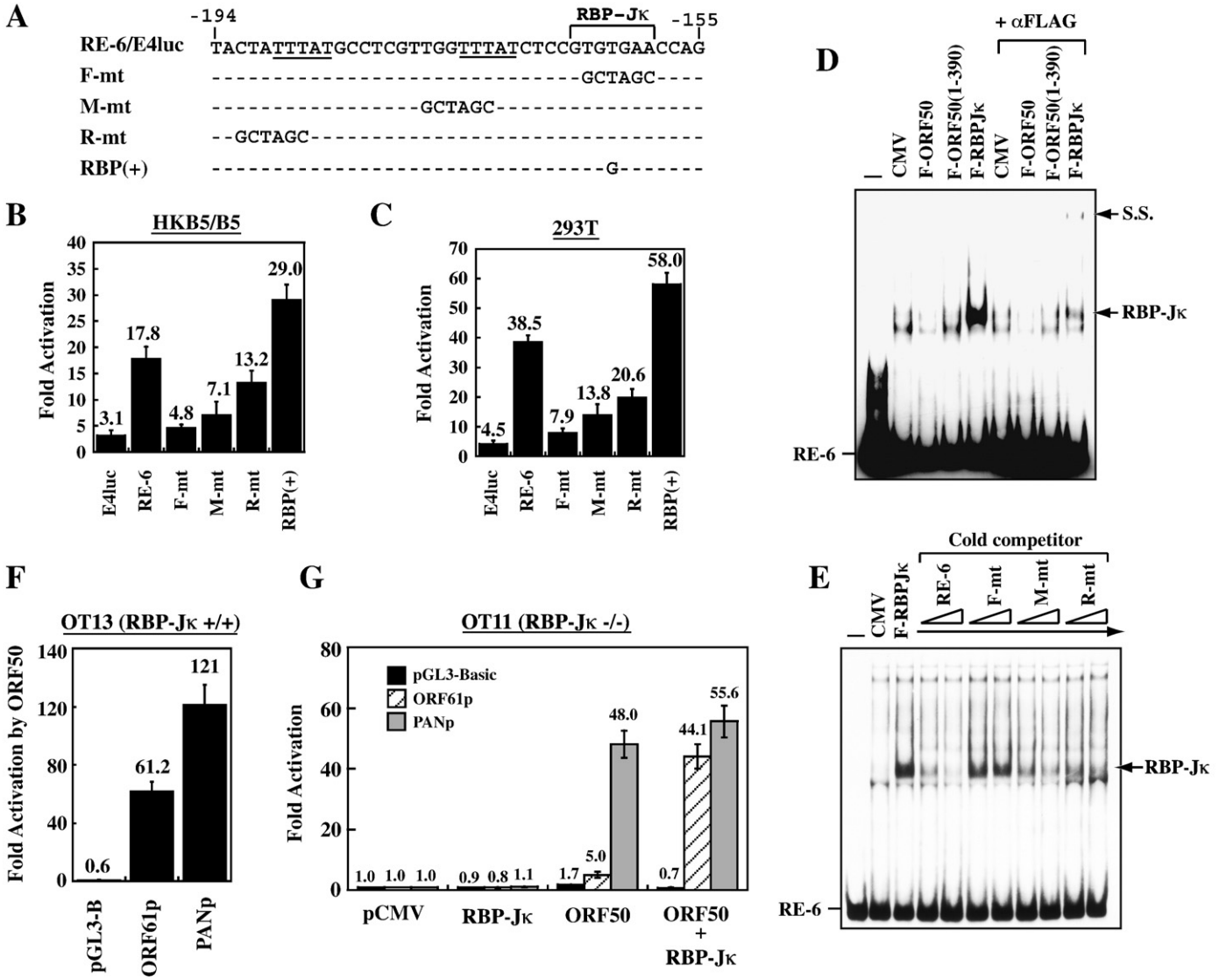

Fig. 4. Involvement of RBP-Jκ protein in the activation of the ORF61 promoter by ORF50. (A) DNA sequence of the wild-type and mutant ORF50 REs of the ORF61p. A putative RBP-Jκ binding site and the mutated sequences in the ORF50 RE of the ORF61p were shown in the diagram. (B and C) The luciferase activities of the indicated reporters in response to ORF50 were examined in HKB5/B5 and in 293T. (D) Binding of the RBP-Jκ protein to the ORF50 RE of the ORF61 promoter. Extracts of HKB5/B5 cells transfected with pCMV, pCMV-FLAG-ORF50, pCMV-FLAG-ORF50(1–390) or pCMV-FLAG-RBPJκ were used in EMSA. The annealed double-stranded oligonucleotide was labeled with biotin-11-UTP using terminal deoxynucleotidyl transferase. Antibody to the FLAG tag was used for supershift tests. (E) Competition assay in EMSA. The indicated cold competitors used in EMSA were in 25 and 50-fold molar excesses over the probe. (F) The reporter plasmid containing the entire ORF61p (−1069 to +130) or PANp (−1875 to +34) was cotransfected with pCMV or pCMV-FLAG-ORF50 into OT13 cells. The ORF50 responsiveness of each promoter was determined by luciferase assay at 48-h post-transfection. Plasmid pGL3-Basic (pGL3-B) represents a control reporter. (G) The indicated effector plasmids, including pCMV-FLAG-RBP-Jκ and pCMV-FLAG-ORF50, were cotransfected with the reporters in OT11 cells. After 48-h post-transfection, activation of the reporters by the effectors was determined.

proteins and DNA was detected by PCR with primers specific for the promoter regions of PAN, ORF57, ORF61, ORF60, ORF62 and LANA as well as for the coding region of ORF65 (ORF65c). Under our ChIP conditions, the ORF50 protein expressed in either HH-B2 or HH-B2 (Tet-On-F-ORF50) was consistently associated with the PAN, ORF57, ORF61 and ORF60 promoters, but not with the ORF62 and LANA promoters, and the ORF65 coding region (Fig. 9). On the other hand, a weak but specific signal for the ORF57p and ORF61p was detected from RBP-Jκ-immunoprecipitated chromatin (Fig. 9).

Discussion

The KSHV ORF50 protein is a multifunctional replication and transcription activator that plays a central role in the switch from viral latency to the lytic cycle (Sun et al., 1998; Wang et al., 2004). Although several distinct transactivation mechanisms operated by ORF50 protein have been elucidated, many early-lytic genes controlled by ORF50 protein still remain to be classified and characterized. Here, we study the transcriptional activation of the ORF61 and ORF60 genes by ORF50 protein. Although the ORF61 and ORF60 genes have similar expression kinetics and related functions in the lytic cycle, we found that ORF50 protein activates these two adjacent genes through different mechanisms. The most striking difference is that ORF50 protein is unable to activate the ORF60p in KSHV-negative cells (Fig. 2). Furthermore, we have identified several specific cellular proteins involved in the regulation of the ORF61p and the ORF60p (Figs. 4, 6 and 8). The functional association between ORF50 and these diverse cellular proteins emphasizes the versatile actions of the ORF50 protein in lytic replication.

The KSHV ORF61 gene encodes the ribonucleotide reductase large subunit (Russo et al., 1996). Based on our Northern analysis (Fig. 1B) and the transcriptional mapping reported by Majerciak et al. (2006),

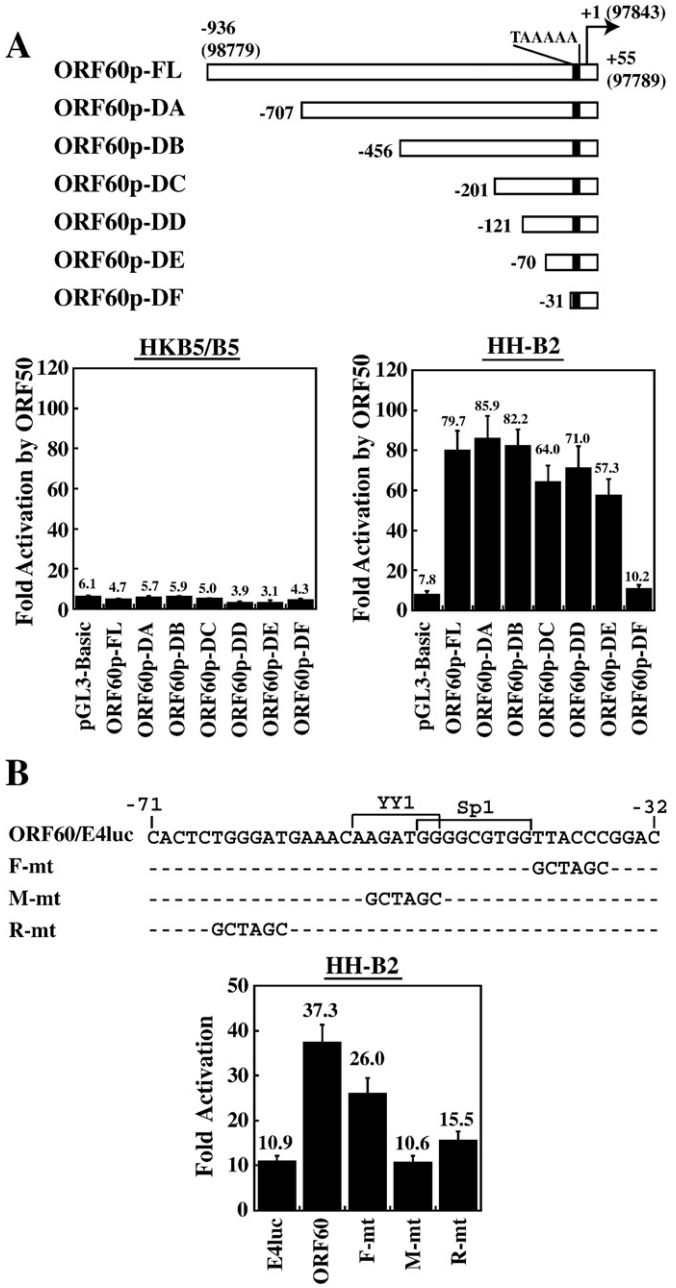

Fig. 5. Mapping the ORF50 regulatory element of the ORF60 promoter. (A) Responses of the deletion constructs of the ORF60 promoter to ORF50 in HKB5/B5 and HH-B2 cells. Schematic diagram of the ORF60 promoter and the size of the ORF60 promoter in each construct were indicated. (B) The regulatory element from −71 to −32 and its mutants in pE4luc were analyzed for their ORF50 responsiveness in HH-B2 cells. Two overlapping binding sites for YY1 and Sp1/Sp3 were shown in the ORF60p(−71/−32) element.

the ORF61 transcript may be a 6.0-kb polycistronic mRNA that contains open reading frames 61, 60, 59 and 58 (Fig. 1A). The transcriptional start site of the polycistronic transcript has been mapped to nucleotide position 100329 of KSHV genome (Figs. 1A and C). The ORF61 promoter contains a consensus TATA box, 5′-TATATAAA (Fig. 1D). Either in KSHV-positive cells or in KSHV-negative cells, ORF50 protein sufficiently activates the ORF61p (Fig. 2). In transient reporter assays, the ORF50 RE of the ORF61p was found in the region between −194 and −155 (Fig. 3), which contains an RBP-Jκ-like recognition site and two A/T-trinucleotide motifs (Fig. 4A). A tandem array of phased A/T-trinucleotide repeats has previously been suggested to mediate the binding by ORF50 and confer the ORF50 responsiveness (Liao et al., 2003). However, we did not detect the direct interaction between ORF50 protein and the ORF61p(−194/−155) element *in vitro* (Fig. 4D). At least four findings in our experiments support that activation of the ORF61p by ORF50 protein is mediated through RBP-Jκ. First, RBP-Jκ protein specifically binds to the putative RBP-Jκ site in the ORF61p(−194/−155) element in EMSA experiments (Figs. 4D and E). Secondly, mutation at the RBP-Jκ site of the ORF61p(−194/−155) element substantially reduces its ORF50 responsiveness (Figs. 4B and C). Thus, binding of RBP-Jκ protein to this recognition site appears to be critical to mediate the ORF50 responsiveness. Thirdly, in RBP-Jκ (−/−) cells, ORF50 protein does not activate the ORF61p unless RBP-Jκ is supplied in *trans* (Fig. 4G). Lastly, the association of RBP-Jκ and ORF50 with the ORF61 promoter in the context of viral genome is detectable by chromatin immunoprecipitation (Fig. 9). Therefore, we conclude that RBP-Jκ is required for ORF50 to activate the ORF61 promoter. In addition to the RBP-Jκ binding site, we notice that sequence flanking the RBP-Jκ site is also essential for a maximal response to ORF50.

The KSHV ORF60 gene encodes the ribonucleotide reductase small subunit (Russo et al., 1996). The ORF60 transcription starts at two positions, nt 97843 and 97845 with a TATA-like sequence, 5′-TAAAAA, present upstream of the start sites (Figs. 1C and D). The ORF60 promoter cannot be activated by ORF50 protein in KSHV-negative cells (Fig. 2). We have mapped the ORF50 regulatory element of the ORF60 promoter in HH-B2 cells. The ORF50 regulatory element is located between −71 and −32, which contains two overlapping binding sites for YY1 and Sp1/Sp3. The YY1, Sp1 and Sp3 proteins are ubiquitously expressed transcriptional factors. YY1 recognizes a conserved 5′-CGCCATNTT and function as either a negative or positive regulator depending upon the promoter context it binds (He and Margolis, 2002; Hyde-DeRuyscher et al., 1995; Shi et al., 1997). Sp1 and Sp3 are structure-related zinc finger proteins, but may have strikingly different functions in cells (Chu and Ferro, 2005; Li et al., 2004). Both Sp proteins bind and act through GC boxes to regulate gene expression of multiple target genes (Chu and Ferro, 2005; Li et al., 2004). The YY1 and Sp1/Sp3 proteins predominantly bound to the ORF60 promoter in EMSA (Fig. 6). Under the lytic condition, formation of the YY1/DNA, Sp1/DNA or Sp3/DNA complexes seems to be reduced (Figs. 6A and B). The down-regulation of YY1, Sp1 and Sp3 at late time points of the viral reactivation may be due to the host shutoff or other regulatory mechanisms. The authentic roles of these cellular proteins binding to the ORF60 promoter in latently or lytically infected cells still need to be further addressed. As shown in Fig. 7, expression of YY1 in cells was reduced in a time-dependent fashion during the lytic cycle. Furthermore, overexpressing YY1 preferentially inhibited the activation of the ORF60p, but not the ORF61p (Fig. 8). These results suggest that YY1 is a repressor for the transcription of certain lytic genes, such as the ORF60 gene, during lytic replication. In addition to the repressive effect by YY1, other viral or cellular proteins directly or indirectly binding to the ORF60 promoter may be required for the activation of the ORF60 promoter. Two candidate complexes, A2 and A3′, detected in EMSA (Fig. 6B) may be important for recruiting ORF50 protein to the ORF60p and then contributing to the ORF50-mediated transactivation.

Due to the fact that ORF50 protein cannot activate the ORF60p in KSHV-negative cells, viral proteins besides ORF50 protein may be required for the activation of the ORF60p during lytic cycle. These specific viral proteins could be latent or lytic proteins or both. We have investigated two lytic transcriptional modulators, ORF57 and K8, in cooperating with ORF50 protein to activate the ORF60 promoter. The ORF57 protein is a homolog of the Epstein–Barr virus (EBV) BMLF1 gene product, which acts as a post-transcriptional modulator (Gupta et al., 2000; Majerciak et al., 2007, 2008). The K8 protein, a homolog of EBV ZEBRA, often cooperates with ORF50 protein to regulate gene expression (Izumiya et al., 2003; Lin et al., 1999). However, neither does the individual protein nor different combinations of ORF57, K8

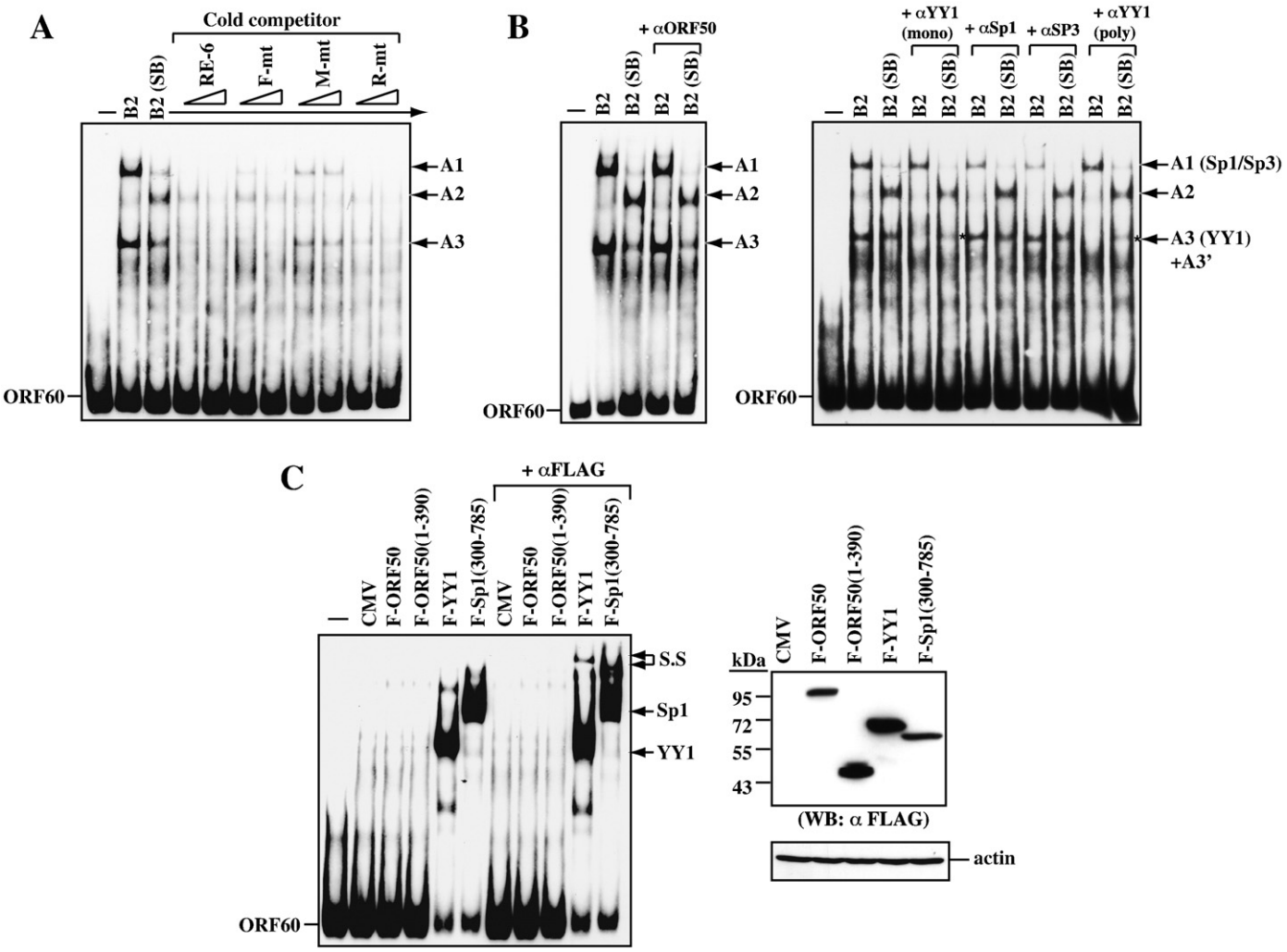

Fig. 6. Binding of YY1, Sp1/Sp3 and unknown proteins to the regulatory element of the ORF60 promoter. (A) EMSA of the ORF60p(−71 to −32) element. The double-stranded ORF60p element (−71 to −32) labeled with biotin-11-UTP using terminal deoxynucleotidyl transferase was served as a probe in EMSA. Protein extracts of HH-B2 cells untreated or treated with sodium butyrate (SB) for 30 h were used in EMSA. The protein/DNA complexes formed in EMSA were indicated. Cold competitors as shown in Fig. 5B were used in 25 and 50-fold molar excesses over the probe. (B) Antibodies to ORF50, YY1, Sp1 and Sp3 were used for supershift analysis in EMSA. Both monoclonal (mono) and polyclonal (poly) antibodies to YY1 were included in the experiment. Asterisk indicates the protein/DNA complex A3′ that co-migrates with the complex A3. (C) Extracts of HKB5/B5 cells transfected with pCMV, pCMV-FLAG-ORF50, pCMV-FLAG-ORF50(1–390), pCMV-FLAG-YY1 and pCMV-FLAG-Sp1(300–785) were prepared for EMSA (left panel). Antibody to FLAG was used for supershift analysis (left panel) and Western analysis (right panel).

and ORF50 significantly activate the ORF60p in HKB5/B5 and 293T cells (data not shown).

In conclusion, we provide here evidence that ORF50 protein regulates the ORF61p and ORF60p via distinct cellular transcription factors. Understanding the diverse regulatory mechanisms operated by ORF50 protein has enhanced our knowledge about the versatile actions of the ORF50 protein.

Materials and methods

Cell cultures and transfections

293T is derived from human embryonic kidney cells transformed with the E1 region of adenovirus and the simian virus 40 T antigen. The 293T cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS). HKB5/B5, an EBV-negative cell clone formed by fusion of HH514-16 cells with 293T cells, was grown in RPMI 1640 medium supplemented with 8% FBS (Chang et al., 2002). HH-B2 cells were cultured in RPMI 1640 medium supplemented with 15% FBS (Gradoville et al., 2000). Mouse RBP-Jκ−/− (OT11) and wild type (OT13) embryonic fibroblast cell lines (Kato et al., 1997) were kindly provided by T. Honjo (Kyoto University) and were grown in high-glucose DMEM supplemented with 10% FBS and 100 U/ml of mouse interferon gamma. HH-B2(Tet-On-F-ORF50) cell clones harboring the doxycycline (Dox)-regulated ORF50 expression system were derived from HH-B2 cells by two selection steps according to user manual of the Tet-On Advanced Inducible Gene Expression System (Clontech). One of the selected clones was used in the study and was maintained in the medium with 100 μg G418 per ml and 400 μg hygromycin per ml. For induction of the exogenous ORF50 protein in the cell clone, doxycycline (Sigma) was added in 1 μg per ml. All transfection experiments were performed in the study using Lipofetamine 2000 (Invitrogen).

Northern blot analysis

HH-B2 cells were treated with 3 mM sodium butyrate in the presence or absence of 200 μg phosphonoacetic acid (PAA) per ml for 12, 24, 36 and 48 h. Total cellular RNAs from the treated HH-B2 cells (1.2×10^7) were prepared with an RNeasy kit (QIAGEN), fractionated on 1% formaldehyde-agarose gels, and transferred to nylon membranes (Hybond-N; Amershann Pharmacia Biotec). The probe

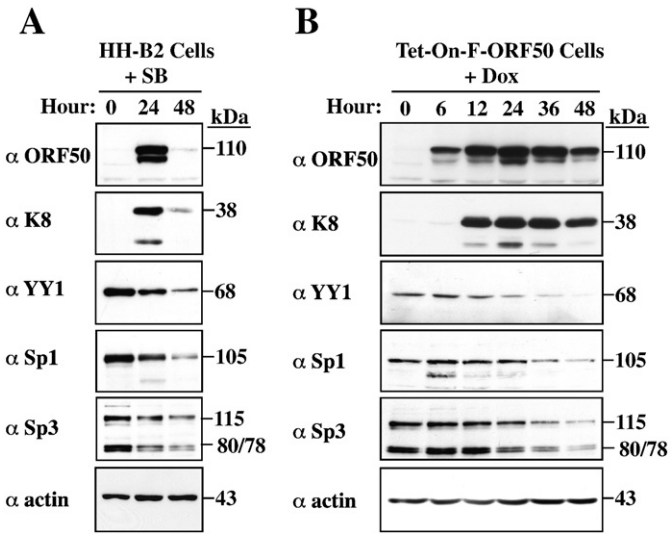

Fig. 7. Down-regulation of YY1, Sp1 and Sp3 expression during KSHV lytic replication. (A) HHB2 cells treated with 3 mM sodium butyrate (SB) for 24 and 48 h were analyzed for the expression of ORF50, K8, YY1, Sp1, Sp3 and actin. (B) Expression profile of the viral and cellular proteins in HH-B2(Tet-On-F-ORF50) cells treated with doxycycline (Dox). HH-B2(Tet-On-F-ORF50) derived from HH-B2 contains an exogenously doxycycline-regulated ORF50 gene. After doxycycline treatment, the cells were harvested at different time points and the protein extracts were prepared for detecting the abundance of ORF50, K8, YY1, Sp1, Sp3 and actin.

corresponding to nucleotides 97527 to 97843 of KSHV genome was synthesized and labeled with 0.1 M digoxigenin (DIG) DNA mix (Roche) by PCR. Hybridization was performed according to the protocol described previously (Chang et al., 2005). After removal of excessive DIG-labeled probe, an anti-DIG antibody conjugated with alkaline phosphatase (1:10,000; Roche) was added. The membrane was then incubated with the enzymatic substrate CSPD (1:100; Roche) to display the specific signals.

Rapid amplification of 5′ cDNA ends (RACE)

The transcriptional start sites were mapped using the GeneRacer kit (Invitrogen). Briefly, HH-B2 cells were treated with 3 mM sodium butyrate for 30 h and total RNA was prepared from the treated cells. Two micrograms of total RNA was treated with calf intestinal phosphatase to remove the 5′ phosphates of truncated mRNA and non-mRNA. After decapping of full-length mRNA by tobacco acid pyrophosphatase, an RNA linker oligonucleotide was ligated to the mRNA by T4 RNA ligase. The first-strand cDNA was obtained by reverse transcription using random primers. Subsequent PCR and nested PCR amplification were carried out using the gene specific primers and the linker primers provided in the kit. The ORF61 specific primers used in the initial PCR and nested PCR were 5′-CTCATTCAT-CATGGAGGGCTGCA and 5′-CGTACTCCACTATAGCCATCTCAG, while the ORF60 specific primers were 5′-CACGACACCGTGGATATTCTCCATG and 5′-TAATAGTGGGCCACGTCGTGGCTGT. The amplified DNA fragments were cloned and sequenced. All clones of the ORF61 gene had the same start site mapped at nt 100329 of KSHV genome (Russo et al., 1996). Six out of ten clones of the ORF60 gene were initiated at nt 97843, while three clones at nt 97845.

Plasmid construction

The DNA fragments of the PAN, ORF57, ORF61 and ORF60 promoters were amplified by PCR from viral genome of HH-B2 total DNA and cloned into pGL3-Basic (Promega) digested with *Hin*dIII (or *Xho*I) and *Nhe*I. The plasmids including pE4luc, pCMV-FLAG-ORF50, pCMV-FLAG-RBPJκ and pCMV-FLAG-ORF50(1–390) were described previously (Chang and Miller, 2004; Chang et al., 2002, 2008). The plasmid pCMV-FLAG-Sp1(300–785) was constructed by inserting a DNA fragment that encodes aa 300 to 785 of Sp1 to pFLAG-CMV-2 (Sigma) with *Eco*RI and *Xba*I. The double-stranded oligonucleotides or PCR-amplified DNA fragments shown in Figs. 3B, 4 and 5B were cloned into pE4luc digested with *Nhe*I and *Xho*I. The plasmid pE2F/E4luc contains two copies of the E2F binding site, 5′-AGTTTCGCGCCCTTTCTCAA, in pE4luc.

Luciferase assays

293T, HKB5/B5, HH-B2, OT11 and OT13 cells (7×10^5) were transfected with a fixed amount (0.8 μg) of plasmid DNA, including the reporter and effector plasmids. The reporter assays were carried out according to manufacturer's protocol for the luciferase reporter assay system (Promega). Activation was calculated as luciferase activity in the presence of activator divided by that in the presence of control vector. The value of fold activation represents at least three independent experiments with duplicate samples in each transfection.

Electrophoretic mobility shift assay (EMSA)

Protein extracts for EMSAs were prepared from HKB5/B5 or HH-B2 cells. The cells were suspended in a lysis buffer (0.42 M NaCl, 20 mM HEPES [pH 7.5], 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg aprotinin/ml). Lysates were centrifuged at 90,000 rpm at 4 °C for 15 min in a benchtop ultracentrifuge and supernatants were harvested and stored at −80 °C. Annealed double-stranded oligonucleotides were end-labeled with biotin-11-UTP using terminal deoxynucleotidyl transferase (PIERCE). Binding reactions contained 10 to 15 μg of

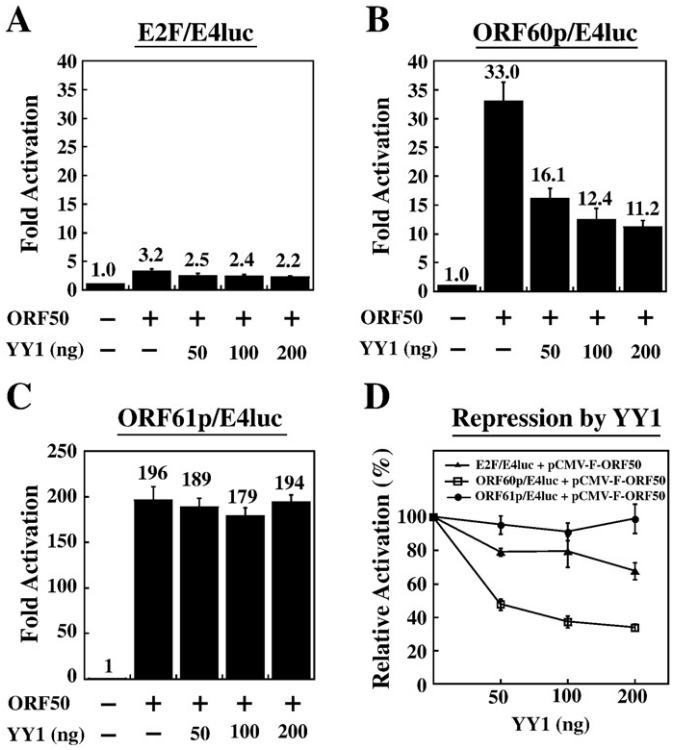

Fig. 8. Inhibition of the ORF60p activation by over-expressing YY1. Fixed amount (0.8 μg) of plasmid DNA, including the reporter and the indicated effectors, was transfected into HH-B2 cells. At 24-h post-transfection, the luciferase activity of the pE2F/E4luc (A), pORF60p(−71/−32)/E4luc (B) and pORF61p(−194/−155)/E4luc (C) was determined. The relative activation of these three reporters by ORF50 in the presence of the increasing amounts of YY1 was summarized in (D).

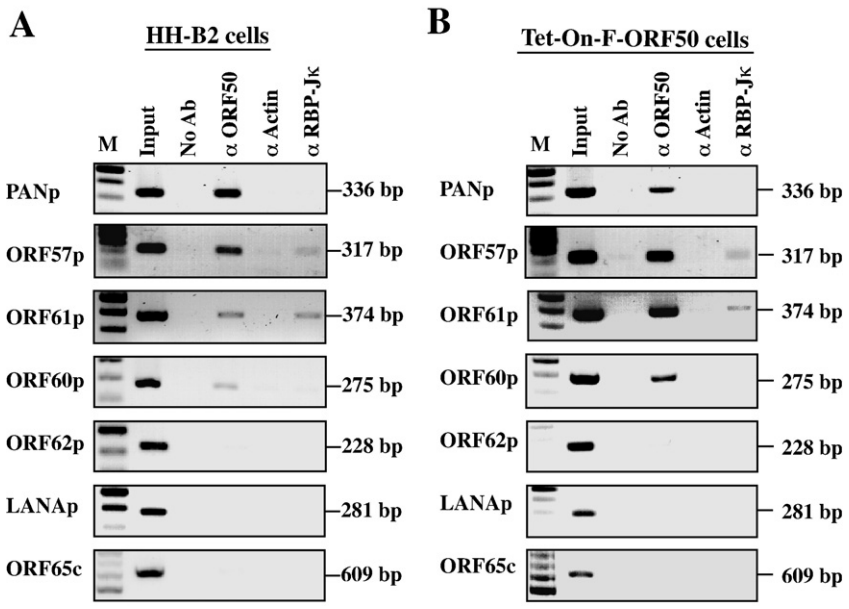

Fig. 9. Association of the ORF50 protein with the ORF61 and ORF60 promoters *in vivo*. The cross-linked chromatin from HH-B2 cells treated with sodium butyrate for 24 h (A) or from HH-B2(Tet-On-F-ORF50) cells treated with doxycycline for 24 h (B) was prepared for chromatin immunoprecipitation. Immunoprecipitation was performed using antibodies to ORF50, actin and RBP-Jκ. Associations between proteins and promoters were detected by PCR using primers specific for the promoter regions of PAN, ORF57, ORF61, ORF60, ORF62 and LANA as well as for the coding region of ORF65. Lanes M, molecular size marker.

protein extract in a solution containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 2.5 μM $ZnSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 15% glycerol and 0.5 μg poly(dIdC) in a total volume of 20 μl. For competition assays, unlabelled competitor DNA was added to the initial reaction mix. The specific antibodies were used for supershift analysis.

Western blot analysis

Cell protein extracts were mixed with 3× sodium dodecyl sulfate (SDS) gel loading buffer and boiled for 5 min before proteins were resolved on 8% to 10% polyacrylamide gel and transferred to PVDF membrane (BIO-RAD). The membranes were blocked in 5% non-fat milk and incubated with diluted primary antibodies for 2 h at room temperature or 4 °C overnight. Anti-rabbit or anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase was used as the secondary antibody. The enhanced chemiluminescence system was used for detection of antibody-antigen complexes. The rabbit polyclonal antibody against ORF50 used in the study was generated using bacterially produced ORF50 protein (aa 1 to 590) as an antigen. Antibodies to K8α (sc-57889; Santa Cruz), YY1 (sc-1703, sc-7341; Santa Cruz), Sp1 (sc-59, Santa Cruz), Sp3 (sc-644, Santa Cruz) and actin (MAB101; Chemicon) were obtained commercially.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as described previously (Chang and Miller, 2004) with minor modification. Briefly, HH-B2 cells (2×10^6) were treated with 3 mM sodium butyrate and HH-B2(Tet-On-F-ORF50) cells were treated with doxycycline. After 24-h treatment, cells were incubated with 1% formaldehyde for 10 min at 37 °C. The cells were harvested, washed with phosphate-buffered saline, resuspended and sonicated. The sonicated cell lysates were precleared with 50 μl of protein A/G agarose slurry containing 20 μg of salmon sperm DNA and 1 mg of bovine serum albumin per ml for 1 h at 4 °C, and then incubated with antibodies to ORF50, RBP-Jκ (sc-28713; Santa Cruz) or actin (sc-1616; Santa-Cruz) at 4 °C overnight with mutation. The immune complexes were precipitated with 60 μl of protein A/G agarose slurry containing 20 μg of salmon sperm DNA and 1 mg of bovine serum albumin per ml for 1 h at 4 °C. After extensive washing steps, the precipitated DNA was extracted. Four percent of the precipitated DNA was used as a template in PCR. Primer sequences used in PCR were following: 5′-GGTGACCCAACATAGTGATTCGGTAG and 5′-AGCGGCAAGAAGGCAAGCAGCGAGC for the PANp; 5′-GGAAGACACTTCCTCTGCATCAACC and 5′-GTCTATCATTGCTTGTACCATGTCC for the ORF57p; 5′-GGAACTGTGCCCAGCGTACAAACC and 5′-AGTACCTTCGTGACCAAGGTCACC for the ORF61p; 5′-GAAATCGATCAACTGAATCCATTGG and 5′-TATCTTAGGGACCTGCTGCTACATG for the ORF60p; 5′- CATATCCACGCCGGCAATGGA and 5′-GCGAACACGCGATAGTCGCGTA for ORF62p; 5′-CCTCGGGAAATCTGGTCTGACAAC and 5′-CCTTGTTTACCTGGCAGGTGAGC for the LANAp; 5′-AAAAGTGGCCGCCCTATCGGCGT and 5′-TGGAAGATGTCCAACTTTAAGGT for the ORF65 coding region.

Acknowledgments

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